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ORIGINAL ARTICLE

Incorporation of bacteriocin in plastic retains activity and inhibits surface growth of bacteria on meat†

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The bacteriocin, nisin, was incorporated into a polyethylene based plastic film and retained activity against the indicator bacteria Lactobacillus helveticus *and* Brochothrix thermosphacta. *Beef carcass surface tissue sections (BCT) topically inoculated with the psychrotrophic spoilage bacterium* B. thermosphacta *were vacuum-packaged both with and without wrapping with the nisin impregnated plastic and held at 4°C. An initial reduction of 2 log_{10} cycles of* B. thermosphacta *was observed with nisin-impregnated wrapped BCT within the first 2 days of storage. After 20 days of refrigerated storage,* B. thermosphacta *populations from nisin impregnated plastic wrapped samples were significantly less than ($P < 0.05$) control vacuum-packaged samples; log_{10} 5·8 vs 7·2 cfu cm^{-2} respectively. Temperature abuse was simulated by shifting inoculated packs from 4°C (after 2 days) to 12°C. Again, by 20 days, the* B. thermosphacta *populations of treated samples wrapped with nisin impregnated plastic were significantly less than ($P < 0.05$) control vacuum-packaged samples; log_{10} 3·6 vs 6·3 cfu cm^{-2} respectively. This work highlights the potential for incorporating antimicrobial peptides with a wider and different range of inhibitory activity directly into plastics of different properties for use in controlling food spoilage as well as preservation to enhance product microbial safety.*

Introduction

Modern red meat processing in the United States relies very heavily upon the use of vacuum-packaging for the preservation of quality and cleanliness of carcass subprimals and further processed meats. The usual means of vacuum-packaging meat subprimals is to place the product in a plastic bag with limited oxygen permeability followed by a mechanical vacuum-package sealing step. As much as 90% of the red meat produced in the USA is vacuum-packaged (American Meat Institute, pers. comm.). Meat products and subprimals held under refrigeration in this way are more shelf-stable than meat held without vacuum-packing. The importance of this preservation technique is increasing as meat export becomes more prevalent and issues of microbial safety continue to escalate. Despite vacuum-packaging technology, the loss of meats to microbial spoilage is very significant (Breidenstein 1986).

Bacteriocins (antimicrobial peptides produced by bacteria which inhibit other closely

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related bacteria) are unique food antimicrobials (for review see Nettles and Barefoot 1993). These agents are generally heat-stable, yet are apparently hypoallergenic and are readily degraded by proteolytic enzymes in the human intestinal tract. One bacteriocin, nisin, is produced by the dairy starter culture organism *Lactococcus lactis*. Nisin has GRAS status (generally recognized as safe) and is approved for use in the US for pasteurized cheese produces as an anti-botulinal agent, as well as in pasteurized liquid egg ingredients. In other nations, nisin is used on a much wider scale as a food antimicrobial.

Nisin and pediocin (a bacteriocin produced by the meat fermentation starter culture bacterium *Pediococcus acidilactici*) have been demonstrated to be active against *Listeria monocytogenes* and other Gram-positive bacterial pathogens on meat surfaces when applied in a liquid form (Chung et al. 1989, Nielsen et al. 1990) and in fluid milk (Jung et al. 1992). Additionally, it has been documented that immobilizing antimicrobials by incorporating into edible alginate gels (Siragusa and Dickson 1992) or spray application of nisin to the surface of meats followed by vacuum-packaging enhances their antimicrobial efficacy (Cutter and Siragusa 1996a, b). Adsorption of bacteriocins to plastic surfaces (Ming et al. 1997) and to siliconized surfaces (Daeschel et al. 1992, Bower et al. 1995a, b) with retention of activity has been accomplished. Combining the bacteriocin directly into a plastic material could provide several advantages as a bacteriocin delivery mechanism. First, only the necessary amount of bacteriocin would be used. Secondly, the agent would not be a direct additive to the food product. Thirdly, if the plastic material were made from an edible and/or biodegradable plastic, environmental advantages would be realized (Gennadios et al. 1997).

Following the packaging process, no other antimicrobial intervention is utilized other than refrigeration. The combination of hurdles from vacuum-packaging, refrigerated storage and bacteriocin inhibition would provide a product with enhanced microbial stability. The objective of this work was to test the hypothesis that incorporation of a bacteriocin directly into a plastic film would retain its antimicrobial activity both *in vitro* and on vacuum-packaged refrigerated meats.

Materials and Methods

Organism

Brochothrix thermosphacta ATCC 11509 and *Lactobacillus* were maintained in storage at −20°C in 75% glycerol. *B. thermosphacta* was grown in tryptic soy broth plus 0·5% w/v yeast extract (TSBYE) at 26°C. *L. helveticus* was grown in *Lactobacillus* MRS broth (Difco, Detroit, Michigan, USA) at 30°C.

Refrigerated storage challenge study

Sections (5 cm × 5 cm) of post-rigor beef carcass surface tissue, *cutaneous truncii*, with intact superficial fascia, were UV-sterilized under a biosafety hood for 15 min (Cutter and Siragusa 1994). Sterilized tissue sections were then inoculated by placing the fascia side down into 10 ml of 10^{-3} dilution of an overnight culture of *B. thermosphacta* ATCC 11509 for 15 min at room temperature. Inoculated sections were treated as follows: treatment W, inoculated tissue sections were prewrapped, envelope fashion, in a 5·5 cm × 11 cm piece of nisin impregnated plastic; treatment U, inoculated tissue sections with no prewrapping; and treatment C, uninoculated with no prewrapping. All treatments were then vacuum-packaged (Hollymatic Model LV10G, Countryside, Illinois, USA) in a standard vacuum-packaging bag (3·2 mil nylon/copolymer bag with oxygen transmission rate at 23°C of 52 cc m^{-2}, Hollymatic Inc., Countryside, Illinois, USA) and held at 4°C. A portion of the samples were shifted from 4°C to 12°C after day 2.

Microbiological analysis

Tissue sections were removed from the packs and placed in a filtered Stomacher bag along with 25 ml of diluent buffered peptone water (BBL, Cockeysville, Maryland, USA) with 0·1% v/v Tween 20 and pummeled for 2 min. *B. thermosphacta* numbers were determined by either Spiral plating (Model D Spiral Plater;

Spiral Biotech, Bethesda, Maryland, USA) and/or spread plating (4 × 250 µl per plate) samples onto plates of STAA agar base with full selective supplement (Oxoid, Basingstoke, UK). Plate counts were converted to the log of colony forming units cm^{-2}. All experiments were replicated four times. Treatment means were separated using a paired Student's *t*-test comparison (InStat2 Version 2.0, statistical analysis package. GraphPad Software, San Diego, California, USA).

Bacteriocin activity assay(s)

Nisin activity of liquid samples was assayed using the seeded lawn overlay spot assay (Siragusa and Cutter 1993). Briefly, TSBYE agar plates were overlayed with 8 ml of semisoft TSBYE agar (0·5% w/v agar) seeded with 8 µl of an overnight broth culture of *B. thermosphacta* ATCC 11509. The seed density was approximately 1×10^6 cfu ml^{-1} of overlay. Plates were scored for zones of inhibition after 16–24 h incubation at 26°C.

Nisin activity of the impregnated and control plastics was determined by placing either a 0·5 cm diameter punched circle or a 1 × 1 cm square directly on the base agar plate and overlaying with the seeded semi-soft agar. Unless specified, all bacteriocin activity assays were conducted with *B. thermosphacta* ATCC 11509 as the indicator organism.

Antimicrobial activity of nisin impregnated plastic against *L. helveticus* was determined on *Lactobacillus* MRS agar and MRS semisoft agar as described above, except that 30°C incubations were used.

The effect of protease treatment on nisin activity of nisin impregnated plastic was determined by adding a 10-µl drop of 1 mg ml^{-1} pronase (Calbiochem, La Jolla, California, USA; catalog no. 53702) on to one side of a 1 × 1 cm square directly on the base agar plate and overlaying with the seeded semisoft agar.

Plastic film preparation

Two hundred grams of powdered low density polyethylene (LDPE, grade 3404, Quantum Chemical Company, Houston, Texas, USA) were dry blended with 8 gm nisin in dried milk solids (Sigma Chemicals, St. Louis, Missouri, USA; catalog no. N5764). The active nisin content of the dried milk solids was 2·5 w/w. The nisin content in the film was therefore 0·1% by weight.

Film was produced using a 19-mm diameter single screw extruder (Brabender, S. Hackensack, New Jersey, USA) with a 0·5 inch blown film dye. The temperature profile was 120/120/120/120°C from the first barrel zone to the dye. Screw speed was 20 rpm. Control film was produced with no nisin and labeled C. Film with 0·1% nisin produced by one pass through the extruder was labeled A. Half of sample A was then reextruded into film and labeled B. Retention time in the extruder under these conditions was approximately 7 min. Film sample B therefore had a total residence time of approximately 14 min in the extruder. A second sample of film was prepared in the same manner, except that the active nisin content was 0·05% by weight. This film was extruded only once, and labeled formula D.

Results

Incorporating a nisin preparation at levels of either 0·1% w/v or 0·05% w/v into the plastic film resulted in retention of antimicrobial activity of nisin (Fig. 1) against nisin sensitive bacteria (Table 1). By testing excised 1 cm^2 sections taken from four evenly spaced sites from the nisin-impregnated plastic (7 cm in length), nisin activity was detectable across the entire nisin incorporated plastic wrap used in the vacuum-packaging experiments after 20 days of refrigerated storage at either 4°C or from the temperature abuse simulation 12°C packs (data not shown). From this data it was concluded that the antimicrobial peptide was evenly distributed throughout the plastic. Protease treatment (Pronase) eliminated the antimicrobial activity of nisin impregnated plastic against an indicator lawn.

Under vacuum-packaged, refrigerated conditions, the test bacterium used in this study, *B. thermosphacta*, is particularly suited to optimal growth (Gardner 1980) and therefore was selected for this purpose. In earlier trials, bacterial populations of inoculated beef carcass

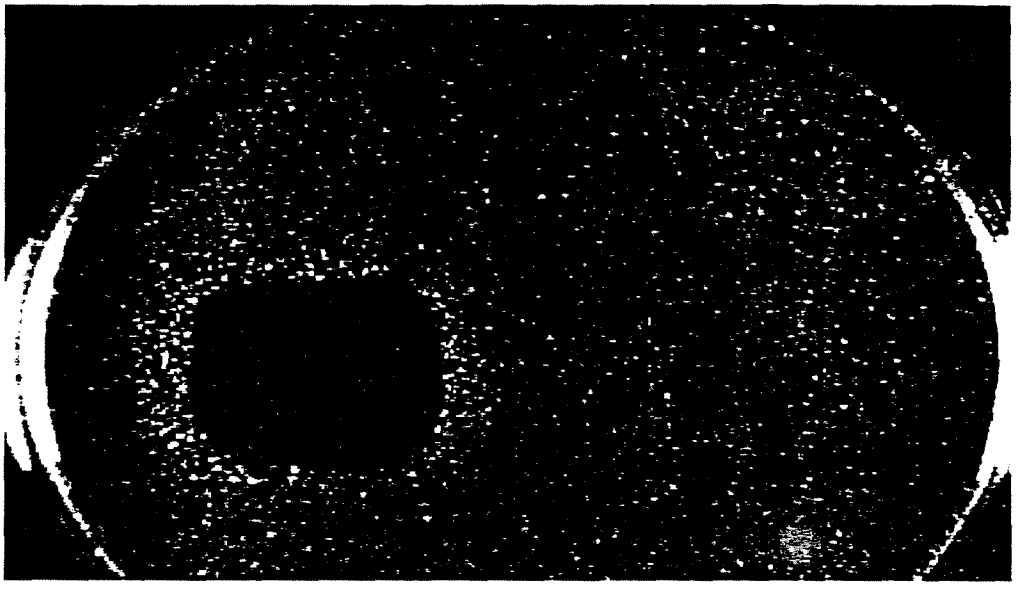

Figure 1. Antimicrobial activity assay of nisin-impregnated plastic. The left hand square is plastic D, formulated to contain 0·05% nisin preparation (Sigma Chemical catalog no. N-5764) and is surrounded by a zone of inhibition. The right hand square is the identical plastic formulation but made without nisin (plastic C); no inhibition is observed. The background lawn of bacterial growth is the meat spoilage bacterium *Brochothrix thermosphacta* ATCC 11509. The plastic squares were outlined to give contrast.

Table 1. Inhibitory activity of nisin impregnated in plastics. Plastics A, B and D contained nisin while plastic C contained no nisin. See Materials and Methods section for explanations of plastic codes and assay protocol. All assays were conducted in duplicate

	Inhibitory activity of nisin				
Indicator strain	Plastic A	Plastic B	Plastic C (control)	Plastic D	In solution
Lactobacillus helveticus	(+)	(+)	(−)	(+)	(+)
Brochothrix thermosphacta	(+)	(+)	(−)	(+)	(+)

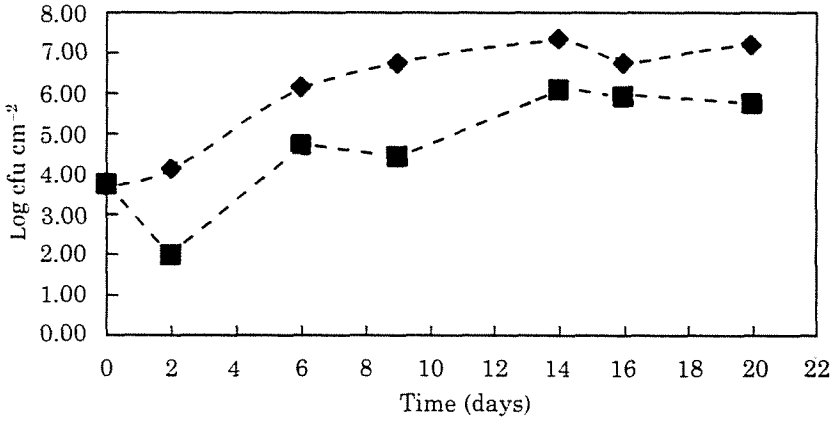

Figure 2. Inhibition of growth of *Brochothrix thermosphacta* inoculated vacuum-packaged beef carcass surface tissue sections with and without nisin impregnated plastic wrapping (plastic C) refrigerated at 4°C. (W=Wrapped in plastic with incorporated nisin before vacuum-packaging (■); U=vacuum packaged only, no nisin-impregnated plastic prewrap (◆).

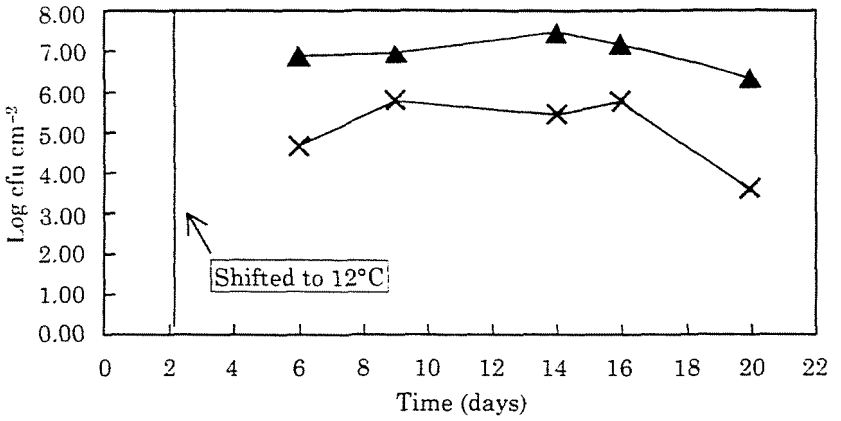

Figure 3. Inhibition of growth of *Brochothrix thermosphacta* inoculated vacuum packaged beef carcass surface tissue sections without and with nisin impregnated plastic wrapping (plastic D) and held at 4°C then shifted to 12°C. (W=Wrapped in plastic with incorporated nisin (plastic D) before vacuum packaging (▲); U=vacuum packaged only, no nisin impregnated plastic prewrap (×).

surface tissue (BCT) that were wrapped in Plastic 'C' (control plastic containing no nisin) were no different than populations from tissue with no wrapping, prior to vacuum-packaging.

B. thermosphacta populations increased to greater than $\log_{10}$ 6 cm^{-2} by day 6 of either the 4°C storage or temperature abuse-simulated storage when simply vacuum-packaged (Figs 2 and 3). However, prior wrapping with nisin-impregnated plastic inhibited *B. thermosphacta* populations to less than $\log_{10}$ 6·09 cm^{-2} by day 20 (the termination day) of either the 4°C storage or temperature abuse simulation (4°C shifted to 12°C) storage. Treatment means from four replications between wrapped and unwrapped at each sampling day were significantly different ($P<0·05$). In the cases of both temperature storage regimens, the inhibitory effect of nisin against *B. thermosphacta* appeared to occur in a short time span of less than 2 days (Figs 2 and 3). This initial decrease in population relative to populations in unwrapped vacuum packaged BCT resulting from the antimicrobial effects of nisin was apparently maintained throughout the storage period of 20 days.

The antimicrobial activity was not extractable from the nisin impregnated plastic by simple room temperature (~23°C) distilled water extractions, even when carried out for as long

Table 2. Extraction of nisin activity from nisin impregnated plastics. Plastics A, B and D contained nisin while plastic C contained no nisin. See Materials and Methods section for explanations of plastic codes and assay protocol. All assays were conducted in duplicate

	Nisin activity detected			
Extraction conditions	Plastic A	Plastic B	Plastic C (control)	Plastic D
Water, RT[a]	(−)	(−)	(−)	(−)
Water, boiling water bath, 5 min	(+)	(+)	(−)	(+)
0·02 NHl, boiling water bath, 5 min	(+)	(+)	(−)	(+)
Saline + 0·5% Tween 20, RT	(+)	(+)	(−)	(+)
Saline + 0·5% Tween 20, boiling water bath, 5 min	(+)	(+)	(−)	(+)

[a]Extracted in water for up to 315 min.

as 315 min. However, mild surfactant extractions at 23°C resulted in leaching of antimicrobial activity (Table 2). Heating the extraction mixture showed extractability of nisin from the plastic material whether under acidic, aqueous or surfactant conditions. Collectively, these data would imply that the nisin was not chemically bound to the polyethylene structure of this plastic formulation.

Discussion

Results of this research clearly demonstrate retention of nisin activity when incorporated into the plastic formulation. Conditions used to produce the film (such as heat and organics) did not eliminate the antimicrobial activity of the nisin. Previous studies have demonstrated the uses of bacteriocins to inhibit bacteria on the surfaces of foods and meats as well as their uses as direct additives (Chung et al. 1989, Nielsen et al. 1990, Jung et al. 1992, Cutter and Siragusa 1996a, b). Subsequent research has focused on the adsorption of bacteriocins to food contact surfaces.

Bower et al. (1995a, b) have reported retention of bacteriocin (pediocin and nisin) activity when coated to siliconized surfaces of varying degrees of both hydrophilicity and hydrophobicity. These authors documented inhibition of susceptible cells of *Listeria monocytogenes* directly added to a siliconized silica surface with adsorbed nisin (Daeschel and McGuire 1995). More recently, Ming et al. (1997) applied bacteriocins (nisin and pediocin) to the inner surfaces of sausage casing by dialysis or to the inner surface of plastic vacuum-packaging bags and demonstrated retention of activity. These same authors also reported using the coated materials to inhibit *L. monocytogenes* growth on ham, turkey breast meat and beef under refrigerated conditions. Both of the aforementioned bodies of research demonstrate that the antimicrobial activity of nisin and pediocin (two bacteriocins from Gram-positive bacteria) was retained upon adsorption to inert surfaces. This work extends that case to include the actual incorporation of a proteinaceous antimicrobial peptide into a petroleum-based plastic with retention of antimicrobial activity.

Shifting a portion of the treated samples to 12°C was carried out to mimic temperature abuse situations that might occur in the food distribution chain. In this case, *B. thermosphacta* was still inhibited by the nisin impregnated plastic.

This study did not address the mode of action or contact mechanism by which the plastic-incorporated bacteriocin inhibited its target bacterium. However, it was demonstrated that inclusion of a surfactant (Tween 20) at low levels would extract the active agent from the plastic at room temperature (~22°C). In a purely aqueous system, heating was necessary for nisin extraction.

In the current work, we demonstrated the efficacy of incorporating low levels of a crudely purified bacteriocin into plastic for inhibiting bacterial growth on a food surface. Further research will be required to establish the parameters for optimal antimicrobial efficiency. Such parameters as plastic formulation, levels of bacteriocin, bacteriocin purity and varying plastic composition will be the focus of further study. The potential for incorporation of antimicrobial peptides (including other foodgrade materials) into plastics that are biodegradable, such as soybean- or cornstarch-based, might offer a myriad of advantages for food safety, shelf-life and environmental disposal concerns.

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